GENERIC RAPID ANALYSIS OF CURRENT AND PROSPECTIVE NERVE AGENTS AND THEIR DEGRADATION PRODUCTS

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ABSTRACT

We have developed a rapid and highly reliable analytical methodology for current and prospective nerve agents and their degradation products. The approach is to augment existing analytical methods with a specific enzymatic transformation step. It utilizes a novel bacterial phosphonate ester hydrolase enzyme (PEH), which degrades the agents' alkali hydrolysis phospho-products (h-agents) to alkylphosphonic acids. Combined with current analytical protocol, which involves testing both the neat and alkali treated sample, the process would generate three distinct phospho-analytes for each agent. Thus, the unique "fingerprint" produced would enable rapid screening and identification of current and prospective nerve agents and their phospho-products using existing instrumental methods (e.g., GC-FPD, GC-PFPD, LC/MS, GC/MS, and GC/MS/MS). For example, we used GC-FPD to ferret out the presence of potential h-agents in samples that were contaminated with other phospho-compounds. This was possible because of the telltale retention time shifts on the chromatograms as the result of the PEH hydrolytic activity. Two critical issues concerning the methodology were addressed in this study: the effectiveness of the alkali to hydrolyze current and prospective nerve agents and the ability of PEH to further degrade the resulting phosphonate esters. All five nerve agents and nine commercially obtained dialkyl alkylphosphonates tested were converted to their respective alkyl alkylphosphonate esters by alkali treatment. This demonstrates the effectiveness of alkali, since the dialkyl alkylphosphonates are assumed to possess much stronger ester bonds than are expected in the leaving groups of any prospective nerve agent. Also, all twenty-six monoalkyl alkylphosphonates tested were effectively degraded by PEH, including those obtained from the above mentioned alkali treatment. The study demonstrates that this new methodology would be an effective analytical approach for current and prospective nerve agents and their phospho-products.

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INTRODUCTION

Current methodologies for the analysis of organo-phosphorous nerve agents (OPNA) have difficulty rapidly detecting and identifying known agents in complex matrices, not to mention unknown agents. A multitude of instrumental techniques are employed in OPNA analysis. The choice of analytical methods for the analysis of unknown samples is based upon intelligence information associated with their collection. If this information is incomplete or incorrect, valuable time is lost performing extensive analysis. Even known agents require confirmatory multi-instrumental analysis for reliable identification requiring larger sample quantities and extensive effort.

Environmental samples could contain a multitude of complex organo-phosphorous compounds due to both biological and industrial processes. Organophosphorus nerve agent analysis in such a sample would be very tedious and time consuming, relying on extensive sample preparation and multiple instrument analysis to reduce background interferents. In addition, because of the labile nature of these neurotoxins, a rapid detection and analysis method is also needed for the agents' phosphonate ester degradation products that are very stable and serve as important markers and biomarkers (Burrows, 1998).

The identification of prospective OPNAs with existing methods poses an almost insurmountable problem because of the tri-substitution at the phosphorus atom and the enormous number of the possible novel agents that can be developed by our adversaries by replacing the existing groups on the phosphorus with various alternatives.

We are developing methods that utilize biotechnology-derived systems to augment existing technologies for improved capability in the analysis of agents and their phospho-products. The focus of the present study is to utilize specific enzymes of microbial origin in order to enhance the scope, speed, and reliability for the detection and analysis of both known and unknown threat agents. The method utilizes sequential degradation of the agents and subsequent analysis of the original substrates and products to generate specific fingerprint from each agent.

As a first step an alkali treatment would hydrolyze nerve agents to their respective alkylphosphonate alkyl ester (h-agent) products. Similar transformations can also be accomplished by Phosphoric triester hydrolase enzymes, such as organophosphorus hydrolase (OPH) (Dumas, 1989, 1990) and organophosphorus acid anhydrolase (OPAA) (Cheng, 1993; DeFrank, 1991).

A bacterial phosphonate ester hydrolase enzyme (PEH) that effectively degrades h-agents to methylphosphonic acid (MPA) has been identified (Elashvili, 2000, 2001). The nerve agent degradation pathway involves alkali or enzyme mediated hydrolysis of the leaving group (fluorine for G-agents: soman, sarin, cyclosarin; mercaptan group for V-agents: VX, Russian VX) followed by hydrolysis of the alkyl-ester bonds by PEH resulting in the formation of methyl phosphonic acid (MPA). The differences in the properties of the different agents and their products can be exploited to generate a unique "fingerprint" from each OPNA. For example, the distinct retention indices on the chromatography columns and volatility characteristics of the agents and their products allow separation by gas chromatography (GC).

Organophosphonate G- and V- nerve agents and their toxic variants possess a phosphonate ester in their structure. Using an enzyme with broad substrate specificity, yet specific for phosphonate esters, allows for quick and reliable identification of suspect compounds in a complex mixture due to their selective degradation. The specific breakdown products allow for tentative but useful identification. The suspect compound(s) can then be interrogated by more rigorous methods ie. GC/MS, LC/MS, GC/MS/MS. Here, we resolve two critical issues concerning the methodology: the effectiveness of the alkali to hydrolyze current and prospective nerve agents and the ability of PEH to further degrade resulting phosphonate esters. The approach is to augment existing analytical methods with the specificity of the enzymatic degradation.

Methods

Partial PEH purification

Burkholderia caryophilli PG2982 was grown in modified MOPS minimal media, which contained (per liter) 7.0 g MOPS, 2.4 g NaCl, 0.6 g Tricine, 0.43 g NH_4Cl, 85 mg $MgCl_2$ $6H_2O$, 40 K_2SO_4, 8.3 mg Thiamine, 1.9 mg $FeSO_4$ H_2O, 1.67 ml Salts (6.0 g $MgCO_4$ $7H_2O$, 3.0 g nitrilotriacetic acid, 1.0 g NaCl, 1.0 g $MnSO_4$ H_2O, 0.5 g $FeSO_4$ $7H_2O$, 0.1 g $CaCl_2$ $2H_2O$, 0.1 g $CoCl_2$ $6H_2O$, 0.1g $ZnSO_4$ $7H_2O$, 20 mg H_3BO_3, 10 mg Na_2MoO_4 $2H_2O$, 10 mg $CuSO_4$ per liter) 4.2 ml 20% glucose, 2.3 ml 40% Na citrate, 2.3 ml 40% K gluconate final pH adjusted to 7.4 with KOH, plus 0.3 mM final concentration of alkali-hydrolyzed GB or dimethyl methyl phosphonate used as the sole phosphorous source. A nutrient agar plate was streaked with *B. caryophilli* and incubated at 30^{O}C for 16-24 hours. A single colony is picked and used to start a seed culture in modified MOPS minimal media and grown 24 hours at 30^{o}C, 200 rpm in a shaking incubator. 8.3 ml of the seed culture, $O.D._{.600nm}$~1.6, was used to initiate 2.4 L cultures in 6 L flasks for 48 hr incubations. Cells were harvested by centrifugation @ 6174 x g, 4^{O}C, 15 minutes. The cells were washed with 15 ml 0.2 M NaCl per 400 ml initial culture, and pelleted at 17,640 x g. Pellets were frozen at –80^{O}C prior to resuspension in 100 mM Tris·Cl pH 8.0, 100mM KCl, 2 mM DTT and lysed using a French press with a 1″ diameter pressure cell 3x times at >1000 psi (American instrument co. Urbana, IL). Cell debris was pelleted at 24,000 x g, 4^{O}C, 30 minutes. Crude extract was stored at -80^{O}C. The chromogenic substrate p-nitrophenyl phenyl phosphonate was used to assay for phosphonate ester hydrolase activity during purification. Unless otherwise noted, all chemicals were of the highest quality available from Sigma chemical company (St. Louis, Mo.).

Chromogenic activity assay

PEH extract 2-10 µl was mixed with assay mix (2mM p-nitrophenyl phenyl phosphonate, 20 mM bis-tris propane, 0.5mM $MnCl_2$) to a total volume of 1 ml. Absorbance was monitored at 405nm at 30sec intervals for 4 min. using a Beckman Du-640 spectrophotometer (Fullerton, CA). Extinction coefficient of 1.74 x 10^4 was determined for p-nitrophenyl phenyl phosphonate.

Partially purified PEH extract

Crude extract was diluted 1:3 with 2 mM DTT and loaded onto a DEAE Sepharose FF (Pharmacia) 2.5 x 15 cm column equilibrated with 20 mM Tris·Cl pH 8.0, 2 mM DTT. The column was washed with 225 ml 20 mM Tris·Cl pH 8.0, 2 mM DTT followed by 280 ml 100 mM KCl, 100 mM Tris·Cl pH 8.0, 2 mM DTT. PEH was eluted with 200 ml 270 mM KCl, 100 mM Tris·Cl pH 8.0, 2 mM DTT, collected in 20 ml fractions.

The active fractions from the DEAE step gradient were pooled and precipitated with $(NH_4)_2SO_4$ at 80% saturation. The precipitated protein was collected by centrifugation at 25,000 x g for 30 min and the pellet resuspended in a minimal volume of 100 mM Tris·Cl pH 8.0, 100 mM KCl, 2 mM DTT and frozen at –80 ^{o}C until use.

PEH vs. phosphonate esters

Nerve agents GB (sarin), GD (soman), GF (cyclosarin), VX, and Russian VX 285mM were alkali hydrolyzed using 10% NaOH w/v at 22^{o}C for seven days after which the solutions were titrated to neutral ph with HCl. Hydrolyzed agents were tested for their ability to be degraded by the ammonium sulfate PEH extract. The reaction consisted of 60µl (50 mM BTP pH 9.0, 1 mM $MnCl_2$, 10 mM substrate) to which 2 µl of PEH extract was added and incubated at 30°C. At appropriate times 10 µl aliquots were withdrawn from the reaction mixture and added to 150 µl methanol to quench the reaction. They were then dried in SpeedVac (Savant, Albertville, Minnesota). They were then derivatized with 50 µl N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-TMS) (Pierce, Rockford, IL) at 100^{O}C for 20 min. After derivatization, 150 µl acetonitrile was added to the sample, vortexed and transferred to microvial inserts. The analysis of 1 µl of the samples was performed ester bonds was monitored using an Agilent 6890 GC-FPD equipped with the autoinjector and a phosphorous filter on ZB-5 column (30m x 0.32 ID, 0.25 µm filament thickness) (Zebron, Torrance, CA). Helium was used as the carrier gas. The setup was as follows: front inlet pressure 8.93 psi, inlet 250^{o}C, initial oven temperature 100^{o}C ramped at 10^{o}C/min to 130^{o}C, then at 15^{o}C/min until 280^{o}C and held at 280^{o}C for 3 min.

A series of alkyl alkylphosphonate compounds were synthesized for us by Dr. Robert Engel (Queens College of CUNY, NY) and Dr. JaimeLee Cohen (Pace Univ, New York, NY). Although their results of ^{31}P NMR reportedly indicated the compounds to be pure, GC-FPD and HPLC with Dionex ED50 conductivity detector (Sunnyvale, CA) analysis in our lab demonstrated the presence of several compounds in some samples (data not shown).

Results and Discussion

Gas chromatography was employed together with a flame photometric detector (FPD) equipped with phosphorus filter (to monitor only phosphorus containing compounds) for the analysis of OPNAs and their trimethyl silylated (TMS) products. Each step of the enzymatic degradation can be monitored by GC-FPD as exemplified by the PEH transformation of hydrolyzed agents to MPA (Figure 1).

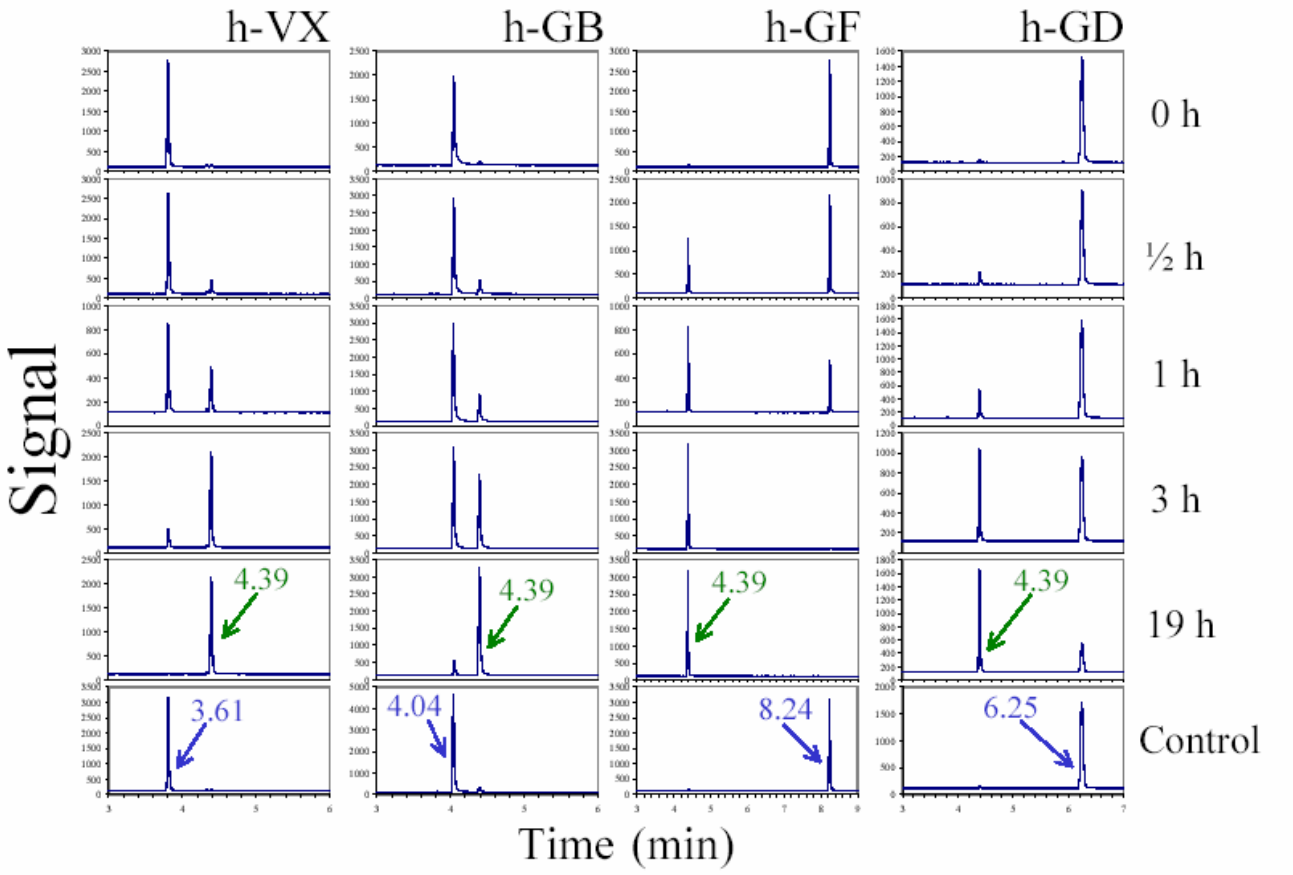

Figure 1. Time course of PEH degradation of hydrolyzed nerve agents monitored by GC-FPD. All the hydrolyzed agents were degraded to methylphosphonic acid (retention time 4.39 min). Control represents the longest hour incubation without PEH.

By hydrolyzing OPNAs to their intermediates and final products, two additional analytes are now available for interrogation. In addition, the second degradation step allows for increased confidence predicated by the specificity of enzymatic degradation. Using this methodology we have developed a retention time database for the OPNAs and their silylated products for GC-FPD (Table 1).

The primary advantage of this methodology is the potential to detect novel threat agents. Alkali is anticipated to be the initial sample-treatment due to the low levels of activity of current organophosphorus hydrolyzing enzymes for V-agents (Dumas, 1989, 1990; Cheng, 1993; DeFrank, 1991) and unpredictability of the effectiveness of the enzyme for unknown OPNAS. Therefore, it was important to confirm alkali's ability to hydrolyze potential nerve agent variants to their alkyl alkylphosphonate esters.

Table 1. The retention times (t_r) of the agents and their silylated products on GC-FPD. Agents were hydrolyzed by alkali treatment followed by degradation with PEH.

Agent Name	t_r Values (ca. min.)		
	Agent	h-Agent	MPA
GB	4.9	4.0	4.4
GD	7.5	6.3	4.4
GF	9.2	7.7	4.4
VX	9.9	3.7	4.4
R-VX	9.7	4.7	4.4

Table 2. Dialkyl alkylphosphonate esters hydrolyzed to monoalkyl alkylphosphonates by alkali treatment

1. Dimethyl methylphosphonate
2. Dibutyl methylphosphonate
3. Diethyl vinylphosphonate
4. Diethyl benzylphosphonate
5. Diethyl benzoylphosphonate
6. Diethyl allylphosphonate
7. Diethyl ethylphosphonate
8. Dimethyl phenylphosphonate
9. Diethyl-4-methylbenzylphosponate

In our experiments, in addition to the five known nerve agents (Table 1), all nine commercially obtained dialkyl alkylphosphonates were converted to monoalkyl alkylphosphonates by the alkali treatment (Table 2). Both ester bonds in dialkyl alkylphosphonates is expected to be stronger than the bonding of the leaving groups of prospective OPNA variants, since the toxicity is dependent on the ease of removal of the leaving groups. Therefore, it is anticipated that alkali would effectively degrade all prospective OPNAs. It is noteworthy that the alkali treatment only removed a single alkyl group from each dialkyl alkylphosphonate while the other alkyl-ester bond was not affected.

The second important consideration in our methodology was whether PEH could degrade a broad range of alkyl-substituted phosphonates. We found that PEH effectively degraded alkali-treated products of GB, GD, GF, VX, and Russian VX nerve agents, but not the products of three similarly treated OP pesticides tested. PEH was tested for its ability to degrade alkyl and aryl esters of other phosphonates in addition to h-agents. All 26 phosphonate esters tested were degraded by PEH (Table 3). The substrate range of PEH appears sufficiently broad for phosphonate esters to allow identification of the prospective OPNAs using our methodology.

The primary benefit of this new methodology is its ability to enable database independent screening for novel variants of OPNAs in rapid and specific fashion. This is demonstrated in Figure 2 using the OPNA simulant dimethyl methylphosphonate (DMMPn). Alkali treatment converts DMMP (t_r =2.92 min.) to methyl methylphosphonate (t_r =3.70 min.) as monitored by GC-FPD, which is subsequently degraded by PEH to MPA (t_r =4.72 min., discrepancy with table 2 values due to different GC setups used). The telltale shift in retention times (Figure 2) because of PEH activity, indicating the phosphonate ester

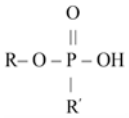

Table 3. t_r values of alkyl alkylphosphonate esters (blue) and their PEH degraded products (green).

R	R′									
	CH_3-	CH_3CH_2-	$CH_3CH_2CH_2$-	$CH_3CH_2CH_2CH_2$-	CH_2CHCH_2-	C_6H_5-	$C_6H_5CH_2$-	$CH_3C_6H_5CH_2$-	CH_2CH-	$C_6H_5C(O)$-
CH_3-	3.7		5.1			8.3				
CH_3CH_2-	4.2	4.9	5.6		5.8		5.6	10.4	4.7	9.6
$CH_3CH_2CH_2$-						8.7				
$(CH_3)_2CH$-	4.4				6.0		5.8			
$CH_3CH_2CH_2CH_2$-	5.9			8.2						
$(CH_3)_2CHCH_2$-	5.5									
$(CH_3)_3CCH(CH_3)$-	6.5					10.5				
C_6H_{11}-	8.2					12.2				
$NO_2C_6H_5$-	11.8					15.8				
$C_6H_5CH_2$-			10.6			13.1				
H- (PEH Products)	4.7	5.5	6.2	7.0	6.2	8.9	6.1	10.6	5.2	9.9

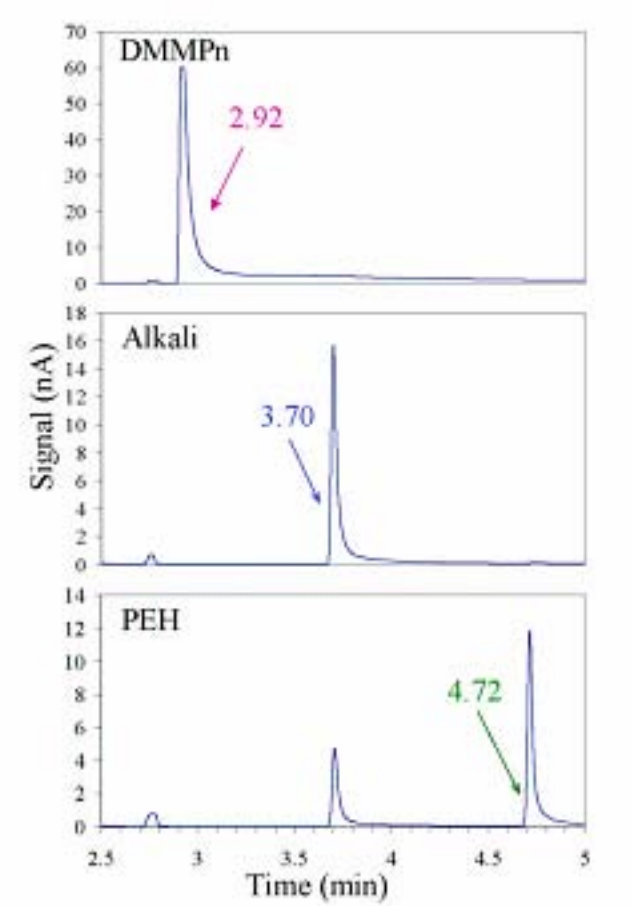

Figure 2. Monitoring DMMPn degradation on GC-FPD: untreated DMMPn (top), and TMS derivatives after alkali treatment (middle) and subsequent PEH treatment (bottom).

substrate (i.e., potential h-agent), allows for greater confidence in the identifying peaks of interest for more detailed analysis (e.g. GC/MS/MS) of potential threats. Not only it enables quick screening, but also it will greatly enhance the agent identification capability by the use of the specific t_r value sets.

Integral with the novel database independent screening is the methodology's ability to identify peaks of interest in complex matrices. The use of GC-FPD with a phosphorous filter allows very low detection limits for phosphorous compounds with greatly reduced background noise by focusing on only phospho-compounds. Some of the novel phosphonate esters synthesized for us were contaminated with unidentified phospho-compounds. In such samples, our methodology allowed easy identification of the synthetic phosphonate ester peaks (ethyl ethylphosphonate and butyl butylphosphonate, identified in blue on Figure 3), which were later confirmed by GC/MS. Without the use of our methodology all the peaks in the chromatogram would require painstaking analysis by several instrumental methods, a very time consuming processes, and perhaps still not yield a definitive answer.

This discovery revealed new approaches to greatly improve the speed and reliability of the analytical methodology of these neurotoxins and their products. While untreated nerve agent analysis can be time consuming and ambiguous, utilizing alkali followed by PEH will produce alkylphosphonic acid that can be rapidly analyzed (e.g. MS/MS). This would enable quick screening of the samples. In addition, the reliability of the existing analytical methodology for these agents can be markedly improved if the analysis of the double treated samples can be included in the protocol together with the untreated and alkali-treated agents. In this case, three phospho-analytes will be generated and their specific indices would greatly enhance the identification capability and results reliability.

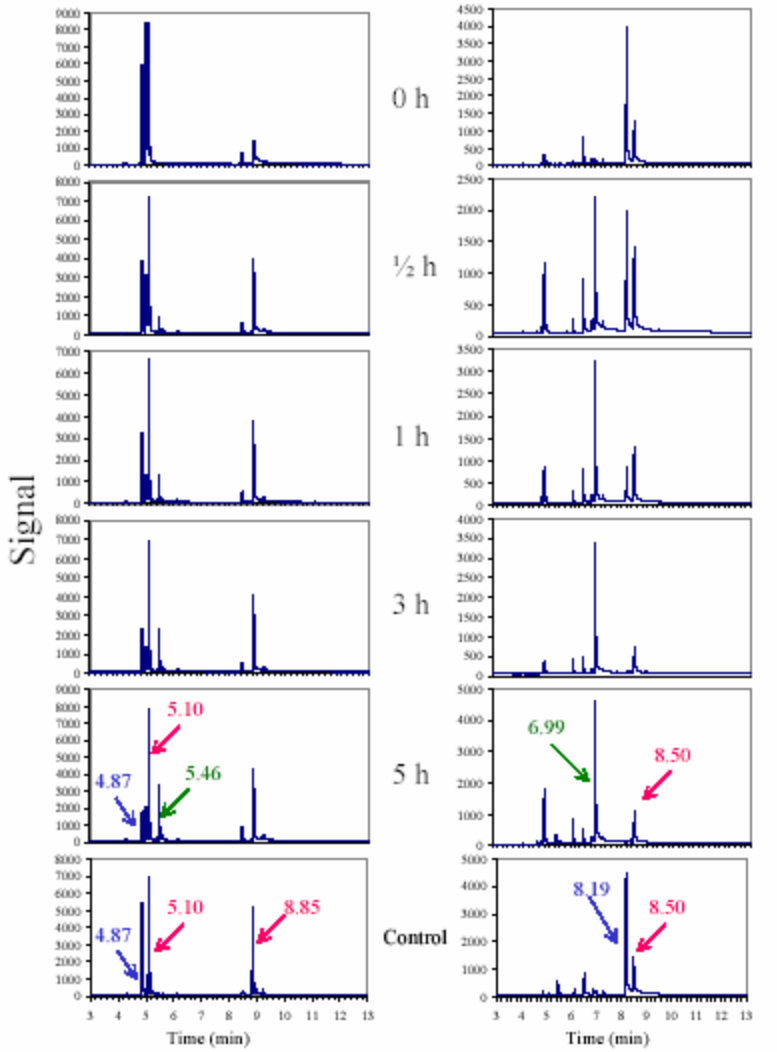

Figure 3. Identification of alkyl alkylphosphonate esters in phospho-compound contaminated samples through retention time (t_r) shifts as the result of PEH hydrolysis (GC-FPD). Enzymatic degradation time courses of ethyl ethylphosphonate (left) and butyl butylphosphonate (right) two synthetic phosphonate esters. Blue depicts substrate, green - product, red - unidentified phospho-compounds

We have demonstrated a novel approach that augments existing methodology for the rapid and highly reliable identification and analysis for the known OPNAs that is predicated by enzyme specificity. In addition, the procedure enables the analysis of the phosphonate ester products of G- and V-type agents' hydrolysis, which are important markers. Furthermore, the approach demonstrates the capability for quick-screening and quantification of novel threat OPNAs and their degradation products and substantial speeding of their identification.

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